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ABSTRACT

DOD Award number DAMD17-03-1-0484, "The Role of c-Src Activation in Prostate Tumor Progression", has as its goal an understanding of the mechanisms of how activation of the protein tyrosine kinase, Src, contributes to prostate tumor progression and how Src regulation in host osteoclasts regulates metastatic growth of prostate tumor cells in the bone. In the first year of work, we developed cell line models required for these studies. Using these cells, we demonstrated that increased Src activity leads to increased expression of vascular endothelial growth factor (VEGF) through a specific transcriptional mechanism involving Src activation of STAT3. We further demonstrate that Src activation leads directly to increased lymph node metastases in orthotopic nude mouse models. These data led to a publication on how Src promotes prostate tumor metastasis. To examine the role of Src in growth of prostate tumor cells in the bone, we have now developed a Src-/- nu/nu strain of mice. Further breeding of these mice is required for tumor studies. Continued studies should provide new insights into role of Src in both the tumor and host environment in promoting prostate tumor cell metastasis.

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INTRODUCTION

DOD Award number DAMD17-03-1-0484, "The Role of c-Src Activation in Prostate Tumor Progression", has as its goal an understanding of the mechanisms of how activation of the protein tyrosine kinase, Src, contributes to prostate tumor progression and how Src regulation in host osteoclasts regulates metastatic growth of prostate tumor cells in the bone. Since the funding for this grant began, Src inhibitors have reached clinical trial, so an understanding of whether or not such inhibitors have efficacy for prostate cancer is becoming even timelier. In addition, recent work by other laboratories has demonstrated novel mechanisms by which Src becomes activated in prostate cancer. Activated Src then contributes to many tumor functions, including resistance to apoptosis and increased expression of angiogenic factors (reviewed by Summy and Gallick, 2003). As stated last year, in prostate cancer cells, we have demonstrated that this activation correlates with increased Vascular Endothelial Growth Factor (VEGF) expression (Kim et al., 2003). However, understanding which functions in tumor progression require Src activation, and the mechanism(s) by which Src contributes to these processes remains unknown, and is a focus of the studies for which this grant was awarded. To complete the tasks we originally proposed, development of prostate tumor cell lines with increased and/or decreased Src expression was required. As discussed below, we have been successful in this endeavor, and have proceeded to finish Task 1 (analysis of VEGF expression). Task 2 (resistance to apoptosis) is now being studied. Task 3 was to examine tumorigenicity in a Src +/+ and Src-/- background. The rationale for use of the Src-/- background is that, in addition to its role in tumor cells, Src plays a pivotal role in osteoclast function, with Src-/- mice suffering from osteopetrosis, due to defective osteoclasts failing to properly mediate bone remodeling (Soriano et al., 1991). Thus, the other goal of studies in this grant is to determine tumorigenicity in this background. To complete Task 3, we needed to breed the Src-/- mice into an nu/nu background. This is necessary because immunocompetent mice will reject human tumor cells. We described our progress in this endeavor last year. This year, we demonstrate that we have been successful in obtaining this strain of mice.

BODY

Task 1

The initial part of **Task 1** was to determine the effects of c-Met and PTEN/MMAC on Src activity and VEGF expression. As stated in last year's progress report, this part of **Task 1** is completed. We determined that decreased c-Met expression resulted in decreased Src activation and decreased VEGF expression (Kim et al., 2003); whereas ectopic expression of PTEN/MMAC had no effect on Src activity and no effect on VEGF expression.

A main component of **Task 1** was to determine the relationship between Src activation, VEGF expression, and tumor progression/metastasis. To complete this task, we were required to generate PC3 and LnCaP human prostate cancer cell clones in which Src was activated. We accomplished this task, as described in last year's report. We demonstrated also that these clones were increased in VEGF expression, as described in last year's Progress Report. This year, we have delineated the mechanism by which Src activation leads to VEGF expression. A paper describing this mechanism (Gray et al., 2005) is now published, and some of that work will be summarized here.

C3.1 Src, Hypoxia and VEGF Expression

Strong evidence has supported a specific role for Src in regulating VEGF expression in normal fibroblasts subjected to hypoxia (Mukhadopathyay et al., 1995). To further test the relationship of Src activation and VEGF expression we have examined VEGF in the cell lines proposed for use in this application. Not surprisingly, PC3 cells are known to increase VEGF expression upon being subject to hypoxia (Fernandez *et al.*, 2001). As shown in Fig. 1, at increasing times in 1% O_2 (hypoxic conditions) Src activity is increased with no changes in expression (not shown). Therefore, Src likely contributes to both constitutive and inducible VEGF expression. We next assessed potential mechanisms by which Src so potently increased VEGF expression. Both HIF-1α and STAT3 have been shown to regulate VEGF expression and are activated by Src. Therefore we investigated the cooperative and/or simultaneous control of the VEGF promoter by these transcription factors (Gray et al., 2005-see APPENDIX). The STAT3 binding site is 86 bp downstream from the HIF-1 binding site (Niu et al., 2002; Wei et al., 2003). In addition, both HIF-1α and STAT3 bind the transcriptional coactivator CBP/p300 (Ebert and Bunn, 1998; Gu et al., 2001; Schuringa et al., 2001; Ray et al., 2002), suggesting that if simultaneous occupancy of the VEGF promoter occurs they may be part of a single transcriptional complex. For these experiments, $CoCl_2$ (100 μM) was used to mimic hypoxia. As shown in Figure 13, this treatment increases Src phosphorylation on tyrosine 418 (comparable to Y416 in chicken Src), indicative of increased Src activation. The SFK selective inhibitor, PP2, blocks this activation (~ 50% decrease relative to control). As also shown in Fig. 13, HIF-1α levels significantly increase in response to hypoxia (~ 4.5 induction). Inhibition of endogenous c-Src activation in PC-3 cells by PP2 drastically reduced HIF-1α levels to below those levels observed at normoxia, again consistent with Src involvement in constitutive and inducible VEGF expression. STAT3 is expressed and phosphorylated in PC-3 cells under normoxic conditions (Fig. 2). However, in cells treated with $CoCl_2$, STAT3 phosphorylation increases substantially, without increases in total STAT3 levels. This increase in STAT3 phosphorylation is inhibited by incubation of cells with the Src inhibitor, PP2.

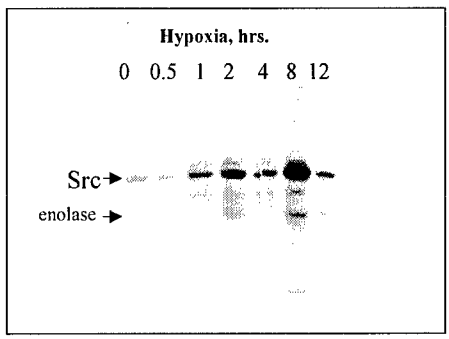

Figure 1. Induction of Src kinase Activity by Hypoxia. PC3-LN4 cells were placed in a hypoxia chamber (1% oxygen) for indicated times. Src kinase activity was measured by the immune complex kinase assay with enolase added as an exogenous substrate.

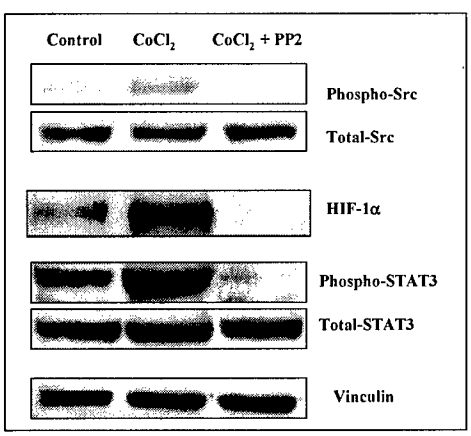

Figure 2. Cobalt Chloride Induces STAT3 phosphorylation and Increases HIF-1α Steady-state Levels. PC-3 cells were treated with $CoCl_2$ with or without PP2 as described in the text. Increased phosphorylation of Src on Y418 (i.e. activation) is required for STAT3 phosphorylation and increased HIF-1α expression.

Expression of Activated Src Increases HIF-1α Levels and Phosphophorylated STAT3

The previous experiments implicated Src activation in the observed changes in HIF-1α and STAT3. To determine more directly if Src activation were causal for HIF-1α and STAT3 phosphorylation the above-described studies were performed in the Src inducible PC-3 subclones (described in last year's progress report) treated with doxycycline (1.5 ng/ml) for 24h. Accompanying the expression of activated Src, HIF-1α levels increased 4.5 fold with respect to controls. Likewise, activated Src led to 2 fold increases of phosphorylated STAT3 (Fig. 3).

Hypoxia or Expression of Activated Src Results in both HIF-1α and STAT3 Binding to the VEGF Promoter

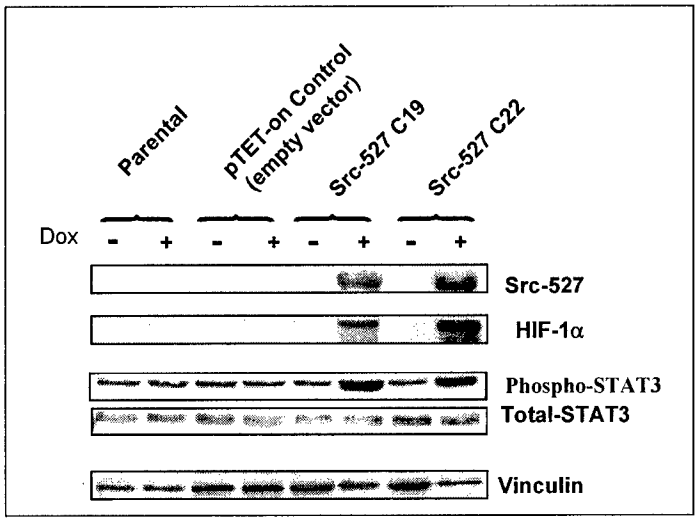

Figure 3. Induction of Src in "Tet-on" Clones leads to Increased HIF-1α Levels and PhosphoSTAT3. Inducible and control clones were described in last year's progress report. Here we show that STAT 3 and Hif phosphorylation increase, which as described below, is critical to increased VEGF expression.

The HIF-1 regulatory region of the VEGF promoter (-985 to –935 bp) contains a STAT3 element 86 bp downstream that has been implicated in VEGF transcription. To determine if Src-dependent increase in HIF-1α levels and phosphorylation of STAT3 results in either or both nuclear factors associating with the VEGF promoter *in vivo,* Chromatin Immunoprecipitation (ChIP) assays were utilized. Fractionated chromatin from controls and $CoCl_2$ treated or Src activated tet-on PC-3 cultured cells were immunoprecipitated with antibody to HIF-1α or STAT3, and the immunoprecipitates isolated. From the isolated DNA a 350-bp region was amplified by PCR for 30 cycles. In normoxic conditions, no detectable HIF- 1α and STAT3 was associated with the VEGF promoter in controls and the untreated tet-on SrcY527F cells in PC-3 cells under normoxic or $CoCl_2$ treated conditions (Fig. 4). By increasing

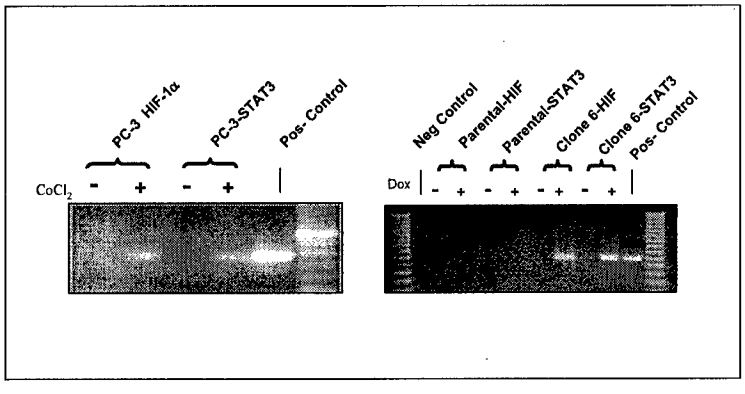

Figure 4. STAT3 and HIF bind at the same 350bp VEGF Promoter Fragment. Chromatin immunoprecipitation assays were performed on extracts from $CoCl_2$ or doxycline- treated cells as described in the text. Both conditions led to increased amplification of the VEGF promoter fragment.

PCR cycles to 50, some HIF-1α binding, but no STAT3 binding was detected (data not shown). Activation of Src PC-3 cells by the addition of doxycycline (1.5 ng/ml for 24 h) showed a considerable increase of HIF-1 and STAT3 associating with the VEGF promoter (Fig. 4). These data demonstrate that activation of endogenous Src or expression of activated Src leads to simultaneous binding of HIF-1α and STAT3 on the VEGF promoter fragment.

Induction of VEGF Expression by Hypoxia or Activated-Src requires HIF- 1α and STAT3 Binding to the Promoter for Optimal Activity

Next we determined the individual effects of STAT3 and HIF-1α on VEGF transcription and protein production. For these experiments, PC-3 cells were transiently transfected with a luciferase reporter plasmid that contained 2.2 kb of the VEGF promoter from cells stimulated by $CoCl_2$ or doxycycline (tet-on Src527 clones) for 24 h. In both cases similar induction of luciferase activity (~ 10 fold) was observed (Fig. 5A). ELISA quantification of VEGF production in the supernatant of cells incubated with $CoCl_2$ or doxycycline to induce activated Src showed concomitant increases in VEGF protein (Fig. 5B). Since the data demonstrate that both HIF-1α and STAT3 bind to the VEGF promoter following Src activation, we examined the absolute requirement of each nuclear factor in VEGF regulation in PANC-1 and PC-3 cells. To interrupt HIF-1α or STAT3 function, we co-transfected with the VEGF-luciferase promoter reporter plasmid alone as a control, or the reporter plasmid and a plasmid directing the expression of a dominant negative HIF-1α or a dominant negative STAT3 plasmid (pCEP4/HIF-1α-DN or STAT3-Y705F respectively). The co-transfected PANC-1 or PC-3 cells where subjected to $CoCl_2$ treatment or activation of Src by the addition of doxycycline (tet-on Src-527 clones). Repression of either HIF-1α or STAT3 function by expression of

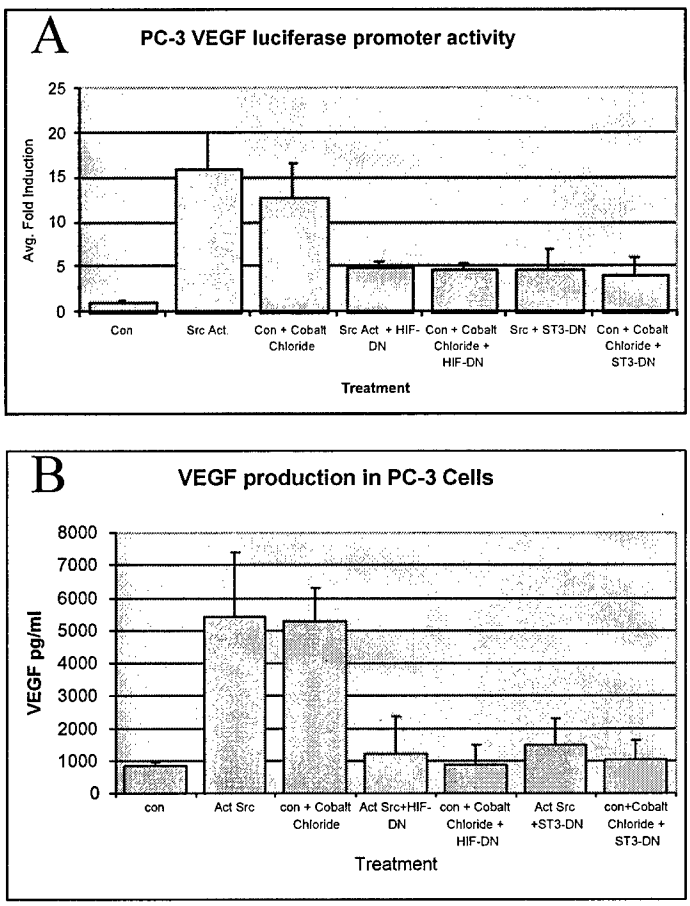

Figure 5. VEGF expression is inhibited by either a Dominant Negative STAT3 or HIF-1α. A. VEGF promoter activity was estimated by luciferase activity as described in the text. Displayed are results of either dominant negative mutant following Src induction in Tet-on clone 6 ("Src-Act.) or following $CoCl_2$ treatment. **B.** VEGF levels in culture supernatants used in A were determined by ELISA.

the appropriate dominant negative construct decreased luciferase activity 3-fold with respect to expression of activated Src alone (Fig. 5A), and reduced VEGF expression to basal levels (Fig 5B). Thus, both functional HIF-1α and STAT3 are required for maximal VEGF activity. In this work, we further demonstrate a transcriptional complex containing STAT3, HIF-1α, p300 and Ref-1/APE (Gray et al., 2005). These data provide a mechanism by which increased Src activity leads to increased VEGF expression, and support our model that the tumor side of the "equation" is likely to result in increased VEGF upon Src activation. Specifically, as diagrammed in Fig. 6, the "normal" response to hypoxic stress is Src activation and increased VEGF transcription/expression. However, when Src becomes ***aberrantly*** activated in prostate cancer (lower part of Figure, the pathway leading to increased VEGF expression (through STAT3) is aberrantly de-repressed. These experiments demonstrate the critical relationship between Src Activation and VEGF expression in prostate tumor cells, and complete **Task 1**.

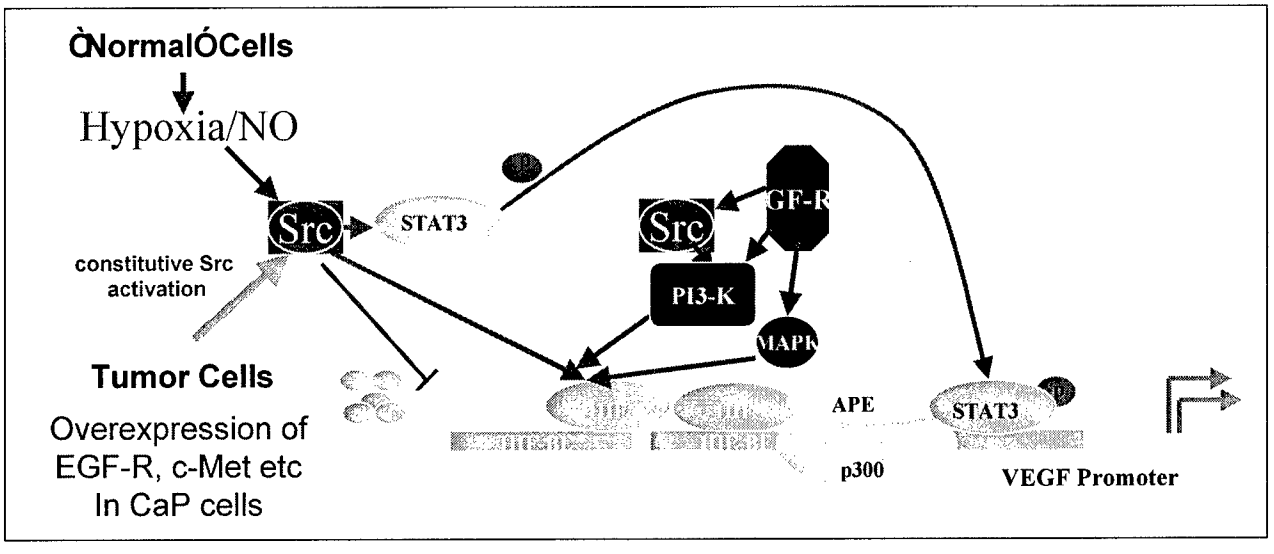

Fig. 6. Model from TASK 1: Src regulates VEGF Expression in Prostate Tumor Cells. In normal cells, hypoxia or NO leads to Src activation, which then leads to STAT3 phosphorylation, increasing VEGF transcription and subsequent production of this angiogenic factor. However, when Src is activated in later stage prostate tumors (lower left), this pathway is deregulated, increasing VEGF expression without the requirement for hypoxia or other stresses. This is the first demonstration of how Src activation can lead to prostate tumor progression, a main goal of studies in this proposal.

Task 2

Task 2 is to determine the role of c-Src expression in inhibition of apoptosis. To initiate this task has required the generation of all the cell lines described in the proposal, now complete. Our first experiment was to examine the relationship of Src activation to anoikis (detachment-induced cell death). We demonstrated that constitutive Src activation leads to resistance of this property associated with metastatic potential. As this experiment has been performed only once, the data will not be presented in this year's progress report. However, we anticipate finishing the task in the next year.

Task 3

Task 3 is to determine the effect of Src expression on growth and metastasis of human prostate tumor cells in orthotopic mouse models. As indicated in the introduction, there are two mouse models to be employed in these studies: "standard" nude mice and Src-/- nude mice, the latter of which need to be developed as described in the initial proposal. We have begun both components of this task. As described in last year's progress report, we demonstrated increased tumorigenicity with the Src-inducible cells. Our initial studies on effects of Src in metastatic potential have focused on the PC3 cells expressing constitutively active Src. Cells (parental or two clones expressing activated Src) were injected into the prostate by precisely the method described in the proposal, and after 30 days, mice were killed (by anesthesia followed by Carbon Dioxide overdose as approved by the VA veterinarian staff). Two different effects of Src were observed as shown in Table 1. When Src was overexpressed 8 fold (-clone 9- more than is

Table 1 *Intra-Prostatic Growth of PC3 and PC3 527 Clones*

	Incidence	Lymph Node Mets (Mean, Range)	Mean Volume (mm^3)	Std Error
PC3	5/5	0, (0)	992.04	+/- 236.12
Clone 20	7/7	3.14, (2-5)	782.89	+/- 178.23
Clone 9	7/7	4.71, (3-8)	1678.13	+/- 332.02

observed in human tumors), there was an increase in both primary tumor growth and incidence of lymph node metastasis. However 3 fold overexpression of Src in clone 20 led to tumor sizes similar to those observed in metastatic variants also led to increased incidence of lymph node metastases, without change in tumor size. These data strongly support a role for Src activation in prostate tumor metastasis. In the next year, we will examine the effect of intratibial injection, a part of **Task 3**.

Development of *src*-/- Nude Mice

A major component of Task 3 was the development of a new strain of mice, *src*-/- nu/nu (hereafter called *src*-/- nude mice) to examine the effects of prostate tumor progression and growth in the bone in a srcless background. In last year's progress report, we indicated success with backbreeding of mice (8 backbreedings into a nude mouse background are required, as described in the initial application, a process that required two years. We have now been successful in generating the first mice of this strain. To develop this strain, we have followed the procedure described by Huang *et al.* (2002) for the development of MMP-9 -/- nude mice. As described in the initial application. Specifically, athymic C57BL/6 mice with wt *src* genes were purchased from Taconic, Germantown, NY. Breeding pairs of *src* +/- mice (identical genetic background) were purchased from Jackson Labs (Catalog # 2277). Mice are genotyped from tail snips. Tail tissues are digested with proteinase K. Genomic DNA (10 μg) purified by multiple extractions with phenol/chloroform:isoamyl-alcohol (24:1) and RNAase treatment (37°C, 1h) is digested at 37°C, overnight with restriction endonucleases (EcoRV/Asp718- for the -0.4 and -1.5 kb and EcoRV/EcoRI for the -8.5 kb u-PAR fragments respectively). Primers to distinguish the wt and mutant alleles, along with specific PCR procedures have been well described by Soriano *et al.*, 1991, and primers and reaction conditions are also detailed on his laboratory website (http://www.fhcrc.org/labs/soriano/protocols/pcrgen.html). Breeding has been

performed in the Animal Facility of the Department of Cancer Biology at this Institution under the direction of the facility's manager, Mr. Elmer Banes. Shown in Figure 7 is analysis of mutant and wild type alleles from some progeny of a cross of a Src+/- male and Src+/- female (with appropriate alleles labeled) after 6 backcrosses into the nude background.

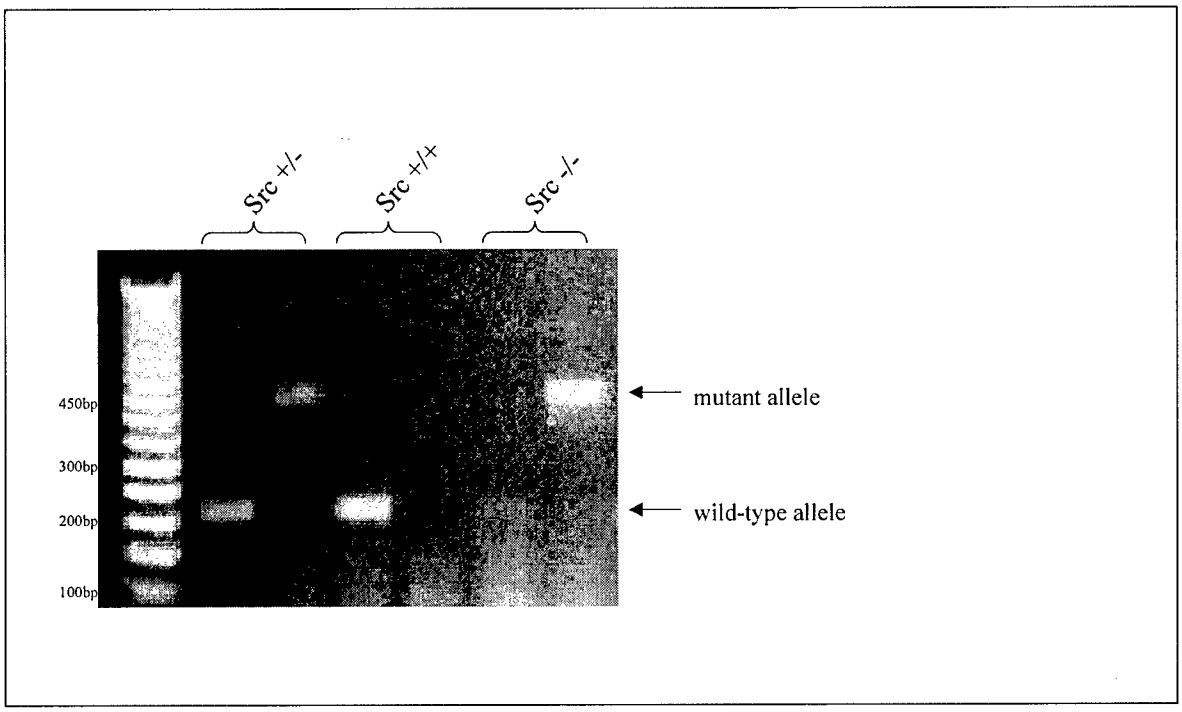

Fig. 7. Analysis of Src Alleles from DNA of Tail Snips of offspring of Src +/- parents after 6 back breedings into an nu/nu background. (A) indicates that wild type and mutant alleles can be identified from offspring. (B) 1 x 10^6 PC3 cells were injected into the flank of one of the nu/nu offspring that formed a large tumor in six weeks. These results demonstrate the ability to grow human tumors in the Src-/- nude mice.

To test that the number of backcrosses were sufficient, 1 x 105 PC3 cells were injected into the flank of one of the mice. Tumor grew comparable to what are observed in Nude mice (if tumors did not grow, it would indicate that the src-/- mice still retain a sufficient immune system to reject the tumor). Thus, we have made excellent progress in **Task 3,** and hope to complete it next year, should breeding of these mice pose no difficulty,

KEY RESEARCH ACCOMPLISHMENTS
(year 1)

- Reduction of c-Met expression reduces VEGF expression in a Src-dependent manner
- Ectopic expression of PTEN/MMAC does not regulate Src activity or VEGF expression
- PC3 human tumor cell subclones were developed that constitutively or inducibly express Src
- Increased Src activity increases VEGF in PC3 cells

- Increased Src in PC3 cells increases tumor growth in nude mice

year 2

- Src activation regulates VEGF expression through STAT3
- STAT3 and HIF-1αform a transcriptional complex on the VEGF promoter in response to activated Src, increasing VEGF expression
- Src activation in PC3 cells leads directly to increased lymph node metastasis in an orthotopic nude mouse model
- *src*-/- nu/nu mice have been successfully established for future tumorigenicity studies

REPORTABLE OUTCOMES

Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, Gallick GE. HIF-1alpha, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene 24: 3110-3120. 2005.

CONCLUSIONS

We have demonstrated that Src activation leads to increased tumor growth of prostate tumor cells, in part by deregulating VEGF expression at the transcriptional level. We demonstrate specifically that Src activation affects outgrowth of lymph node metastases, but not size of the tumor in the prostate (when Src is overexpressed to a physiologically relevant degree). Our results provide strong evidence that Src is important in progression of prostate cancer, and that inhibition of Src may be of therapeutic benefit. As new Src inhibitors are in clinical trial, these results provide impetus for clinical trials on prostate cancer.

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HIF-1α, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas

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Hypoxia stimulates a number of pathways critical to cancer cell survival, including the activation of vascular endothelial growth factor (VEGF) transcription. In normal fibroblasts, hypoxia-induced activation of the protein tyrosine kinase, Src, is required for VEGF expression. We show here in both pancreatic and prostate carcinoma cell lines cobalt chloride (used to mimic hypoxia) -induced VEGF expression requires Src activation and leads to increased steady-state levels of HIF-1α and increased phosphorylation of signal and transducer of transcription 3 (STAT3). STAT3 and hypoxia-inducible factor (HIF)-1α bind simultaneously to the VEGF promoter, where they form a molecular complex with the transcription coactivators CBP/p300 and Ref-1/APE. Expression of activated Src from an inducible promoter is sufficient to increase VEGF expression and form these STAT3/HIF-1α-containing promoter complexes. Inhibition of DNA binding by expression of either STAT3 or HIF-1α dominant negative mutants significantly reduces VEGF expression. These data suggest that the binding of both STAT3 and HIF-1α to the VEGF promoter is required for maximum transcription of VEGF mRNA following hypoxia.
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Keywords: HIF-1α; VEGF; STAT3; Src; hypoxia; angiogenesis

Introduction

Rapidly proliferating cells require a continual supply of oxygen and nutrients. When tissue growth surpasses the ability of the surrounding vessels to deliver these requirements, oxygen deprivation, or hypoxia, occurs. Hypoxia initiates a variety of cellular responses including activation of proto-oncogenes and subsequent angiogenesis. This process is critical to growth and metastasis of tumor cells (Folkman, 1992, 2002; Ellis and Fidler, 1996). The most studied of the peptides that promote angiogenesis is vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) (Senger *et al.*, 1983; Leung *et al.*, 1989; Shweiki *et al.*, 1992; Namiki *et al.*, 1995). VEGF is a potent angiogenesis promoter and is overexpressed in numerous human cancers including pancreatic, colon, and prostate carcinomas (Takahashi *et al.*, 1995; Itakura *et al.*, 1997; Jackson *et al.*, 1997). VEGF enhances migration, proliferation, and reverses senescence of endothelial cells, thus stimulating blood vessel formation (Guo *et al.*, 1995; Senger *et al.*, 1996; Watanabe *et al.*, 1997).

Many signal transduction pathways have been implicated in promoting VEGF expression. Activation of the protein tyrosine kinase, c-Src, and/or its downstream mediator phosphatidyl-inositol 3-kinase (PI3K), have been extensively studied for their roles in promoting VEGF expression (Mukhopadhyay *et al.*, 1995; Eliceiri *et al.*, 1999; Alvarez-Tejado *et al.*, 2001). In both tumor and normal cells, hypoxia causes a rapid increase in the activity of the Src (Namiki *et al.*, 1995; Ellis *et al.*, 1998). Transfection of cells with the activated form of Src (v-Src) resulted in elevated levels of HIF-1α and VEGF expression under normoxic conditions, with further activation occurring under hypoxic conditions, while downregulation of c-Src reduces both VEGF expression, tumor growth and tumorigenicity in murine models (Fleming *et al.*, 1997; Jiang *et al.*, 1997; Staley *et al.*, 1997; Wiener *et al.*, 1999).

The precise signaling pathway(s) by which Src regulates VEGF expression by hypoxia remain(s) unclear. Src and PI3-kinase activation lead to increased expression and stability of the transcription factor hypoxia-inducible factor-1α (HIF-1α), which plays a

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critical role in regulating VEGF transcription (Forsythe *et al*., 1996; Jiang *et al*., 1997; Chan *et al*., 2002; Karni *et al*., 2002). In normoxia, the von Hippel-Lindau protein (pVHL) rapidly degrades subunits of HIF-1α by targeting it for the ubiquitin–proteasome pathway (Iliopoulos *et al*., 1996; Maxwell *et al*., 1999; Ivan *et al*., 2001). HIF-1α protein levels and transcriptional activity are negatively regulated by O_2-dependent hydroxylation (Iliopoulos *et al*., 1996; Ivan *et al*., 2001; Lando *et al*., 2002). Under hypoxic conditions, activated HIF-1α translocates to the nucleus and dimerizes with HIF-β (also known as aryl hydrocarbon receptor nuclear translocator (ARNT)) and associates with the coactivators p300 or CBP. The HIF-1 heterodimer binds to a hypoxia response element (HRE) in the promoter and enhances transcription of the downstream gene (Forsythe *et al*., 1996; Jiang *et al*., 1996).

More recently, signal and transducer of transcription 3 (STAT3) activation has been shown to mediate VEGF transcription (Niu *et al*., 2002b; Wei *et al*., 2003). Src phosphorylates STAT3, and this phosphorylation is essential for the ability of v-Src to transform cells (Yu *et al*., 1995; Turkson *et al*., 1998). Activation of gene transcription by STAT3 also requires its dimerization, but unlike HIF-1α, STAT3 forms homodimers and under some circumstances heterodimers with STAT1 (Bromberg and Darnell, 2000; Calo *et al*., 2003). Latent STAT3 is cytoplasmic but phosphorylation leads to dimerization, nuclear translocation, and activation of target genes including VEGF (Schaefer *et al*., 1999; Niu *et al*., 2002b; Wei *et al*., 2003; Yahata *et al*., 2003). Hypoxic activation of STAT3 has not been previously determined, although the STAT family member STAT5 undergoes hypoxic activation (Joung *et al*., 2003).

Interestingly, while both HIF-1α and STAT3 have been shown to regulate VEGF expression and are activated by Src, potentially cooperative control of the VEGF promoter by each of these factors has not been investigated. The STAT3-binding site is 86 bp downstream from the HIF-1-binding site (Forsythe *et al*., 1996; Wei *et al*., 2003). In addition, both HIF-1α and STAT3 bind the transcriptional coactivator CBP/p300, suggesting that if simultaneous occupancy of the VEGF promoter occurs, they may be part of a single transcriptional complex (Arany *et al*., 1996; Schuringa *et al*., 2001). We report here for the first time that exposure of cells to cobalt chloride ($CoCl_2$), which mimics the hypoxic induction of Src, results in activation of the VEGF promoter through binding of both HIF-1 and STAT3. Inhibition of the binding of either transcription factor significantly reduces VEGF expression. In addition, we show that HIF-1α physically associates with STAT3, CBP/p300, and Redox effector factor-1/apurinic/apyrimidinic endonuclease (Ref-1/Ape). We demonstrate that optimal transcriptional control of the VEGF promoter requires binding of both HIF-1 and STAT3, and these factors are part of a large transcriptional complex coordinated in part by the coactivators CBP/p300 and Ref-1/APE.

Results

Activation of Src, HIF-1α, and STAT3 by hypoxia

To determine the effect of hypoxia upon Src activation in pancreatic and prostrate cancer cells, cultured cells were exposed to $CoCl_2$ (100 μM) as a mimic to hypoxia (Minchenko *et al*., 1994), or $CoCl_2$with the Src inhibitor PP2 (10 nM) for 18 h. As shown in Figure 1a, hypoxia increases Src phosphorylation on tyrosine 418, indicative of increased Src activation in the pancreatic cancer cell line PANC-1 and prostrate cancer cell line PC-3 (~2-fold increase relative to control in both cell lines), while the Src inhibitory PP2 blocks this activation (~50% decrease relative to control). Next, the effect on HIF-1α was determined. As also shown in Figure 1a, while HIF-1α is expressed at normoxia in PANC-1 and PC-3 cells, its levels significantly increase in response to hypoxia (~4.5–8-fold induction). Inhibition of endogenous c-Src activation in PANC-1 and PC-3 cells by PP2 drastically reduced HIF-1α levels to below those levels observed at normoxia (Figure 1a). STAT3 has also been implicated in VEGF expression (Niu *et al*., 2002b), but its potential activation by hypoxia is yet to be determined. As shown in Figure 1a, STAT3 is expressed in PANC-1 and PC-3 cultured cells under

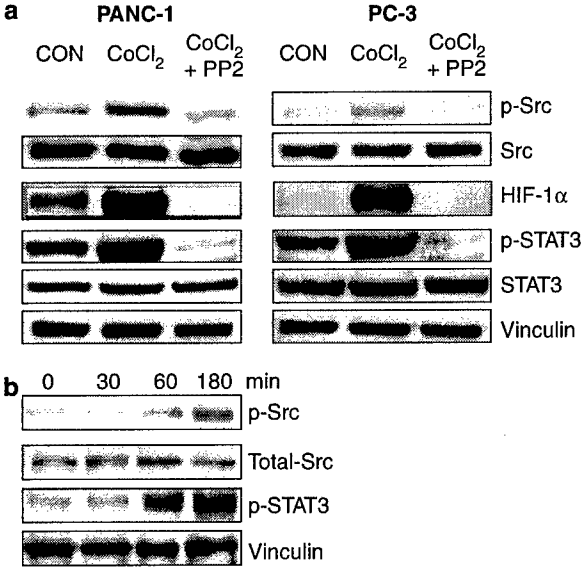


Figure 1 Requirement for Src activation on $CoCl_2$ induced increased expression of HIF1α and phospho-STAT3. (**a**) PANC-1 and PC-3 cells were grown in the presence and absence of the Src inhibitor, PP2, as described in Materials and methods. Cells were stimulated by $CoCl_2$ (100 μM) for 90 min and all cells were lysed as described. Immunoblotting was performed with phospho-Src 418, total Src, HIF-1α, -phospho-STAT3, or -STAT3 antibody. Immunoblots were reprobed with an antibody to vinculin as a loading control. (**b**) PANC-1 cells were grown in 1% cMEM and treated with $CoCl_2$ (100 μM) and lysed for Western Blot analysis at the time points indicted. Immunoblotting was performed with phospho-Src 418, total Src, or phospho-STAT3 antibody. Immunoblots were reprobed with an antibody to vinculin as a loading control

normoxic conditions. While basal levels of phosphorylated-STAT3 are observed in both PANC-1 and PC-3 cell lines, treatment with $CoCl_2$ results in increased STAT3 phosphorylation, without increases in total STAT3 levels. This increase in STAT3 phosphorylation is inhibited by incubation of cells with the Src inhibitor, PP2. To ensure that these molecular changes were not due to $CoCl_2$ treatment but reflective of changes occurring after 'true' hypoxia, cells were incubated for 3 and 6 h in a hypoxic chamber (1% O_2, 99% N_2). Similar changes were observed to those seen with $CoCl_2$ (not shown). While these results support previous observations that Src is an upstream activator of STAT3 (Schaefer *et al.*, 1999), we performed a time course activation of Src and STAT3 by $CoCl_2$ to ensure that STAT3 activation did not precede that of Src. As shown in Figure 1b, Src and STAT3 activation occurs concomitantly following 60 min of $CoCl_2$ stimulation.

Expression of activated Src increases HIF-1α levels and phosphophorylated STAT3

The previous experiments implicated Src activation in the observed changes in HIF-1α and STAT3. To determine more directly if Src activation is causal for increased steady state levels of HIF-1α and STAT3 phosphorylation in PANC-1 and PC-3 cells, subclones in which activated chicken SrcY527F could be induced by doxycycline were created as described in Materials and methods. Incubation of these PANC-1 and PC-3 subclones with doxycycline (2 ng/ml) for 24 h induced activated Src expression (Figure 2). Accompanying the expression of activated Src, HIF-1α levels increased substantially over controls (PANC-1 ~8-fold induction; PC-3 ~4.5-fold induction). Likewise, activated Src led to increased levels of phosphorylated STAT3 (PANC-1 and PC-3, ~2–3-fold increase) relative to controls (Figure 2).

Src-mediated nuclear translocation of STAT3 and HIF-1α

Activation of HIF-1α and STAT3 results in nuclear translocation of each factor. To determine if Src activation led to this translocation, immunohistochemistry was performed on Src-activated cells utilizing an anti-HIF-1α antibody and an antibody, which recognizes activated STAT3 (phospho-STAT3). Prior to Src activation in PANC-1 tet-on Src-527 cells, both HIF-1α and phospho-STAT3 were diffusely located throughout the cell with little staining of either factor observed in the nucleus (Figure 3a and b). Src activation resulted in complete nuclear translocation of all HIF-1α (Figure 3a) and phospho-STAT3 (Figure 3b). To determine if HIF-1α affected STAT3 nuclear translocation or *vice versa*, cells were transiently transfected with an HIF-1α or an STAT3 dominant negative plasmid (pCEP4/HIF-1α-DN or STAT3-Y705F, respectively), each of which interferes with DNA binding of its target factor. The dominant negative HIF-1α protein, which has a mutated DNA domain, has previously been shown to reduce HIF transcription by competing with endogenous HIF-1α for its required transactivation partner HIF-1β. Interruption of STAT3 transactivation was accomplished through expression of a DNA-binding-defective STAT3 protein, which prevents STAT3 DNA binding and subsequent transcription when dimerized with endogenous STAT3 (Forsythe *et al.*, 1996; Kaptein *et al.*, 1996; Yahata *et al.*, 2003). In PANC-1 cells in which activated Src was induced, expression of HIF-1α-DN caused a distinct diffusion of its target factor from the nucleus to the cytoplasm (Figure 3a), but did not affect phospho-STAT3 nuclear localization (Figure 3b). Likewise, prevention of phospho-STAT3 association with its respective DNA element caused a similar nucleus to cytoplasm diffusion (Figure 3b), but did not affect HIF-1α localization (Figure 3a). Morphologic changes characteristic of expression of activated Src were observed, but this morphology was not altered by transfection with the respective dominant negative constructs. These data show that expression of activated Src results in HIF-1α and activated STAT3 nuclear translocation, and retention of each factor is not dependent upon translocation and/or retention of the other. We also show that presumptive prevention of dimerization and DNA binding by each targeted factor results in subcellular relocalization only of that specific factor. STAT3 and HIF-1α localization to the nucleus

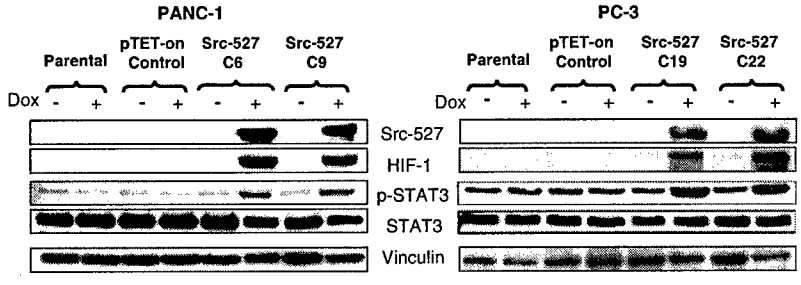


Figure 2 Effect of induction of activated Src on HIF1α expression and STAT3 phosphorylation. Parental, empty vector controls, or tet-on Src-527-expressing cells lines were grown and incubated with and without doxycycline (2 ng/ml) 24 h prior to cell lysis as described in Materials and methods. Expression of activated Src was ascertained by immunoblotting with an avian-specific Src antibody (EC-10). In addition, immunoblotting with HIF-1α, phospho-STAT3, and total STAT3 was performed. Immunoblots were reprobed with an antibody to vinculin as a loading control

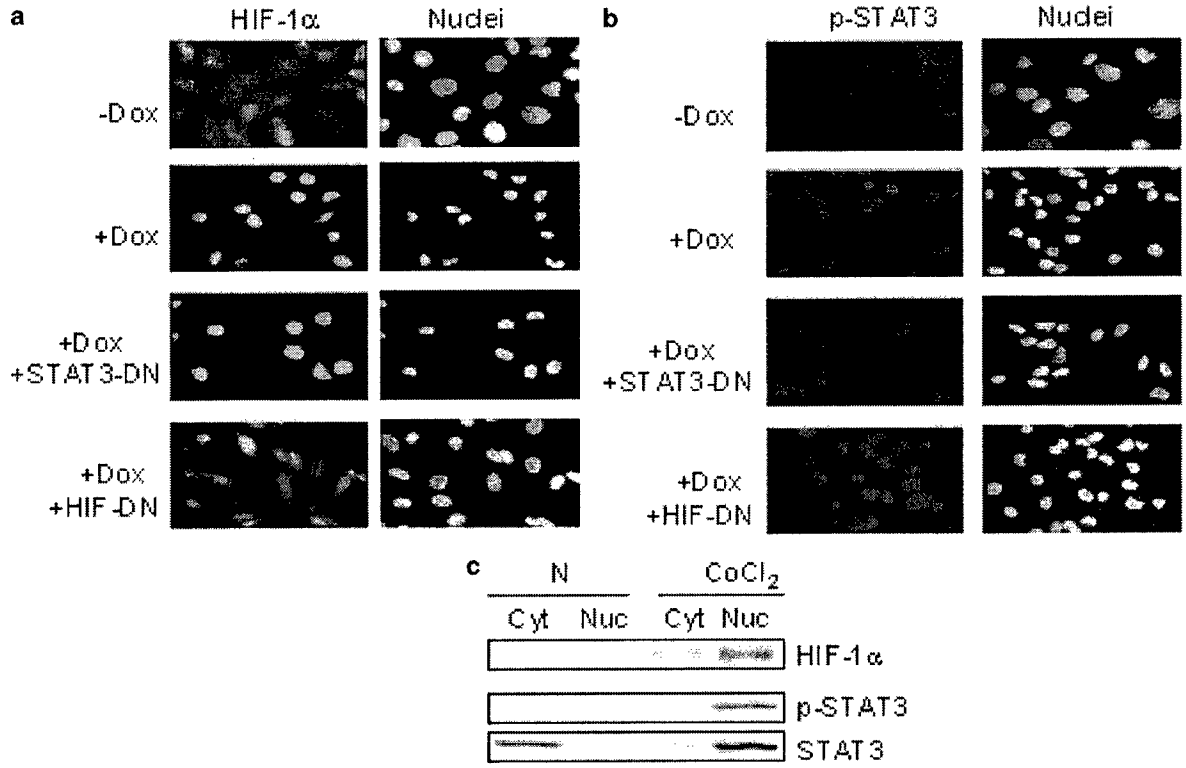


Figure 3 Effect of Src activation on subcellular localization of HIF1α and phospho-STAT3. Immunofluorescence of HIF-1α (**a**) and phosphotyrosine-STAT3 (**b**) was performed in PANC-1 tet-on Src-527 cells with and without expression of activated Src. Cells were seeded in an eight-chamber slide and grown in complete media for 24 h prior to transfection with control DNA or the indicated dominant-negative plasmid. Following transfections, cells were allowed a 16 h recovery period before stimulation. (**c**) Western blot analysis on cytosolic and nuclear faction in PANC-1 cells grown in 1% cMEM and either untreated (normoxia) or stimulated with $CoCl_2$ (100 μM) for 6 h. Subcellular extracts were prepared as described in Materials and methods. Immunoblotting was performed with anti-HIF-1α, or -phospho-STAT3 antibody

following $CoCl_2$ treatment were confirmed by fractionation experiments. Prior to $CoCl_2$ treatment, unphosphorylated STAT3 is found exclusively in the cytosol. Following 6 h $CoCl_2$ treatment, only phospho-STAT3 is observed and only in the nuclear fraction (Figure 3c). HIF-1α expression is low prior to $CoCl_2$ treatment and after treatment expression is primarily in the nucleus.

Hypoxia or expression of activated Src results in both HIF-1α and STAT3 binding to the VEGF promoter

Both HIF-1 and STAT3 have been implicated in mediating VEGF transcription (Niu *et al*., 2002b), but the simultaneous binding of each transcription factor to the VEGF promoter by Src activation or hypoxia has not been examined. The HIF-1 regulatory region of the VEGF promoter (−985 to −935 bp) contains a STAT3 element 86 bp downstream that has been implicated in VEGF transcription (Forsythe *et al*., 1996; Wei *et al*., 2003). To determine if Src-dependent increase in HIF-1α levels and phosphorylation of STAT3 results in either or both nuclear factors associating with the VEGF promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were utilized. Fractionated chromatin from controls and $CoCl_2$-treated (to mimic hypoxia) or Src-activated tet-on PANC-1 and PC-3 cultured cells were immunoprecipitated with antibody to HIF-1α or STAT3, and the immunoprecipitates isolated. From the isolated DNA, a 350-bp region was amplified by PCR for 30 cycles. In normoxic conditions, no detectable HIF-1α and STAT3 was associated with the VEGF promoter in controls and the untreated tet-on SrcY527F cells in PANC-1 (Figure 4a) and PC-3 cells (Figure 4b) or in PANC-1 and PC-3 cells treated with $CoCl_2$ (Figure 4c). By increasing PCR cycles to 50, some HIF-1α binding, but no STAT3 binding was detected (data not shown). Activation of Src in either the PANC-1 and PC-3 cell lines by the addition of doxycycline (2 ng/ml for 24 h) showed a considerable increase of HIF-1α and STAT3 associating with the VEGF promoter (Figure 4a, b, lanes 7 and 9). Similar binding of HIF-1α and STAT3 was observed in PANC-1 and PC-3 cells incubated with $CoCl_2$ (Figure 4c, lanes 3 and 5, 7 and 9, respectively). These data demonstrate that hypoxia (as mimicked by $CoCl_2$) and expression of activated Src both lead to simultaneous binding of HIF-1α and STAT3 on the VEGF promoter fragment.

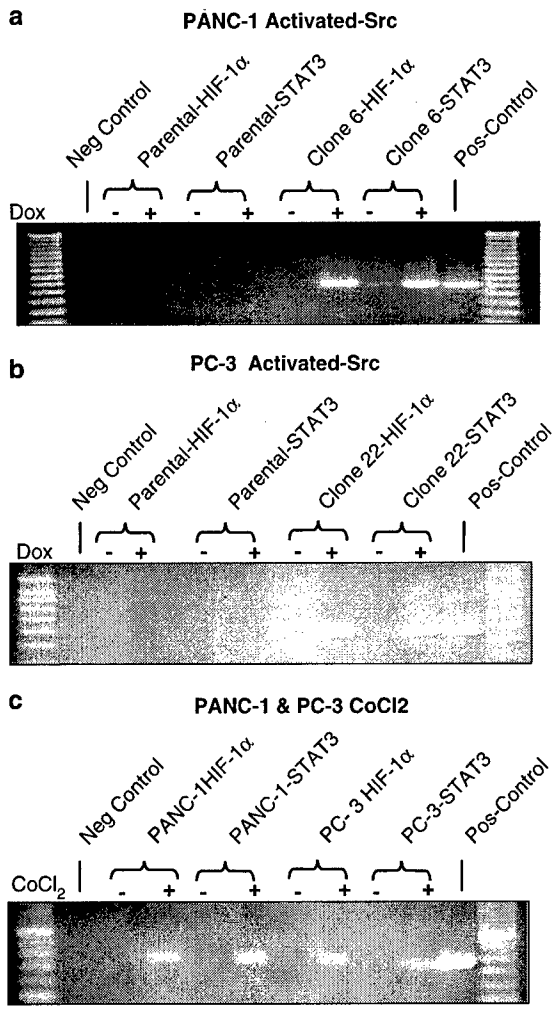


Figure 4 Coimmunoprecipitation of HIF-1α and STAT3 on the VEGF promoter. ChIP assay with HIF1α or phospho-STAT3 antibodies were performed as described in Materials and methods. Cells were grown in the presence and absence of either $CoCl_2$ or doxycycline. The immunoprecipitated DNA was purified as described in Materials and methods and the region from −1386 to −1036 bp of the human VEGF promoter was amplified by PCR (30 cycles). PCR products were run in a 1.2 % gel and visualized by ethidium bromide. (**a**) ChIP from PANC-1 cells; (**b**) ChIP from PC-3 cells (**c**) Effect of $CoCl_2$ on HIF-1α and phospho-STAT3 binding. ChIP assays were performed as described above on lysates from PANC-1 and PC-3 cells (approx 1.0×10^{10} cells each) treated with or without $CoCl_2$ (100 μM) for 16 h

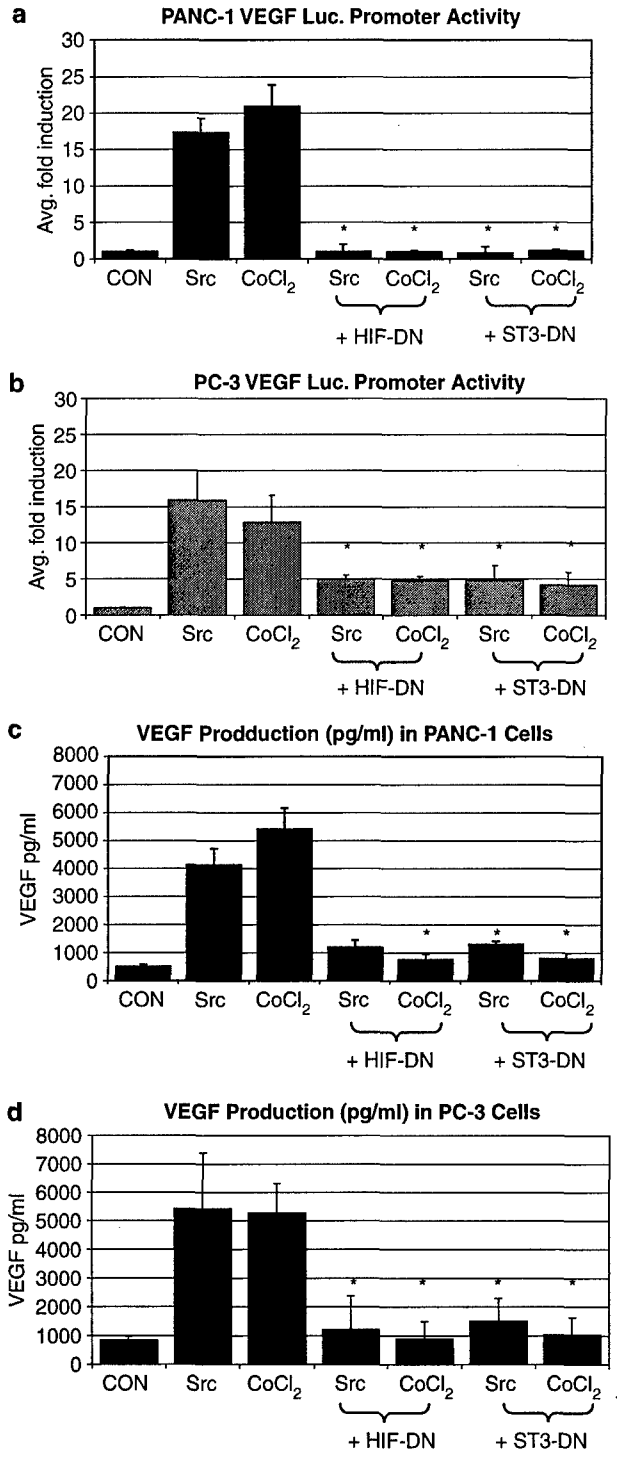
Induction of VEGF expression by hypoxia or activated-Src requires HIF-1α and STAT3 binding to the VEGF promoter for optimal activity

Next, we determined the individual effects of STAT3 and HIF-1α on VEGF transcription and protein production. For these experiments, PANC-1 and PC-3 cells were transiently transfected with a luciferase reporter plasmid that contained 2.2 kb of the VEGF promoter from cells stimulated by $CoCl_2$ or doxycycline (tet-on Src527 clones) for 24 h. $CoCl_2$ and induction of activated Src in tet-on Src527 clones each induced similar levels of luciferase activity (~10-fold) relative to controls (Figure 5a, b, first two columns). ELISA quantization of VEGF production in the supernatant of cells incubated with $CoCl_2$ or doxycycline to induce activated Src showed concomitant increases in VEGF protein (Figure 5c, d, first two columns). These data support previous studies that hypoxic activation of Src (as mimicked by $CoCl_2$) is a central regulatory factor in VEGF production (Minchenko *et al.*, 1994). Coincubation of doxycycline-treated Src tet-on Src527 clones with $CoCl_2$ showed no significant change in promoter activity and VEGF production compared to individual treatment (data not shown), further suggesting that Src is central to VEGF expression. Since our data demonstrates that both HIF-1α and STAT3 bind to the VEGF promoter following Src activation, we examined the absolute requirement of each nuclear factor in VEGF regulation in PANC-1 and PC-3 cells. To interrupt VEGF transcription by these factors, we cotransfected with the VEGF-luciferase promoter reporter plasmid alone as a control, or the reporter plasmid and either a dominant negative HIF-1α (pCEP4/HIF-1α-DN) or STAT3 dominant negative expression plasmid (STAT3-Y705F). The cotransfected PANC-1 or PC-3 cells were subjected to $CoCl_2$ treatment or activation of Src by the addition of doxycycline (tet-on Src-527 clones). Repression of either HIF-1α or STAT3 function by expression of the appropriate dominant negative construct decreased luciferase activity three fold with respect to expression of activated Src alone (Figure 5a, b), and reduced VEGF expression to basal levels (Figure 5c, d). Thus, both functional HIF-1α and STAT3 are required for maximal VEGF activity. Recently, STAT3 has been reported to form heterodimers with STAT1 (Bromberg and Darnell, 2000; Calo *et al.*, 2003). While STAT1 is expressed in both PANC-1 and PC-3 cells, it is not phosphorylated under hypoxic conditions (data not shown). Thus, the transcriptional changes observed in VEGF expression are likely to result predominantly from STAT3 homodimers.

The VEGF transcriptional complex contains HIF-1α, p300, STAT3, and Ref-1/APE

The HIF-1-binding site in the VEGF promoter is at −985 to −935, while the STAT3-binding site is 86 bp downstream at −849 to −842 (Forsythe *et al.*, 1996; Niu *et al.*, 2002b; Wei *et al.*, 2003). Owing to the proximity of the two sites, we sought to determine if HIF-1α and STAT3 were components of the same transcriptional complex. As both HIF-1α and STAT3 have been reported to associate with transcriptional coactivator CBP/p300, we used nuclear extracts from PANC-1-uninduced and activated Src-induced cells to determine if HIF-1α, p300, and STAT3 coimmunoprecipitated (Ebert and Bunn, 1998; Gu *et al.*, 2001; Schuringa *et al.*, 2001; Ray *et al.*, 2002). As shown in Figure 6, nuclear extracts immunoprecipitated with antibody to p300 (Figure 6a) or HIF-1α (Figure 6b), coimmunoprecipitated with STAT3, with increased association in extracts from Src-induced cells. Extracts immunoprecipitated

with an anti-phospho-STAT3 antibody and probed with HIF-1α also showed a similar increase in association following Src activation (Figure 6c). Recently, it has been reported that Ref-1/Ape, which is a cotranscription factor in addition to being a DNA repair enzyme, may be a necessary component of HIF-1α transcription complex (Carrero *et al*., 2000; Ziel *et al*., 2004). To ascertain if Ref-1/APE is part of the STAT3/HIF-1α/p300 complex, nuclear extracts were immunoprecipitated by Ref-1/APE and probed for STAT3 coimmunoprecipitation (Figure 6d). These data demonstrate that HIF-1α, STAT3, CBP/p300, and Ref-1/APE are all likely present in the same complex on the VEGF promoter.



Discussion

Angiogenesis is a vital step in the continued growth and progression of tumors and intratumoral hypoxia is a principal regulator of angiogenesis (Semenza, 2002). One of the most critical proangiogenic peptides induced by hypoxia is VEGF, and expression of VEGF correlates with poor prognosis in a number of human tumors (Ishigami *et al*., 1998; Fontanini *et al*., 2002).

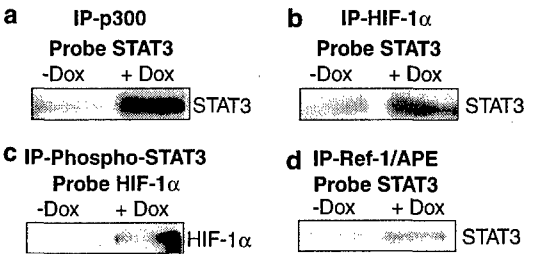


Figure 6 Coimmunopreciptation of transcription factors after Src induction. Nuclear extracts were prepared from untreated and doxycycline treated (2 μg/ml, 24 h) PANC-1 tet-on Src-527 and immunoprecipitations were performed as described in Materials and methods. (**a**) Immunoprecipitation with an anti-p300 antibody and blot probed for STAT3. (**b**) Immunoprecipitation with an anti-HIF-1α antibody and blot probed for STAT3. (**c**) Immunoprecipitation with an anti-phospho-STAT3 antibody and blot probed for HIF-1α. (**d**) Immunoprecipitation with an anti-Ref-1/APE antibody and blot probed for STAT3

Figure 5 Dependence of activated HIF1α and phospho-STAT3 on transcription and expression of VEGF. Luciferase activity was determined in (**a**) PANC-1 cells and (**b**) PC-3 cells with or without expression of the indicated dominant negative mutant utilizing a luciferase reporter plasmid containing 2.2 kb of the human VEGF promoter. Cells were seeded in 12-well plates (0.25×10^6 cells/well) and grown in complete media. Following a 24 h recovery period, cells were transfected with 200 ng of VEGF reporter plasmid and either 1.3 μg of pCEP4 (control wells), 1.3 μg of HIF-1α-DN plasmid (HIF-1α dominant negative), or 1.3 μg of STAT3-DN (STAT3 dominant negative) plasmid as indicated. Cells were treated 16 h later with $CoCl_2$ (100 μM) or doxycycline for 24 h at which time the medium was removed and stored at −80°C for ELISA and cells were lysed. Aliquots (20-μl) of each sample were analysed for luciferase expression and expressed as RLU. Relative luminescence was calculated as average luciferase activity (RLU)/protein concentration. All transfections were performed in triplicate. Asterisks denote significant differences ($P<0.05$) from columns with activated Src and treatment with $CoCl_2$ (**c**) VEGF expression in culture supernatants of PANC-1 cells and (**d**) PC-3 cells. From the above-described conditions, the medium was removed from cells and stored at −80°C. VEGF in the culture supernatants was determined by ELISA as described in Materials and methods. Asterisks denote significant differences ($P<0.05$) from columns with activated Src and treatment with $CoCl_2$

VEGF expression is believed to be predominantly regulated by the transcription factor HIF-1α, which in turn is regulated by signaling pathways initiated by molecules including growth factors and cytokines (Zelzer *et al.*, 1998; Thornton *et al.*, 2000; Liu *et al.*, 2002; Mayerhofer *et al.*, 2002; Semenza, 2003). Alterations in these signal transduction cascades in tumor cells can lead to constitutive expression of VEGF in normoxic conditions, primarily through activation of the PI3-kinase, MAP kinase, and Src pathways (Harris, 2002; Semenza, 2003). Activation of the PI3-kinase and MAP kinase pathways leads to increased rates of HIF-1α translation, although this may be cell type dependent (Semenza, 2003; Bardos and Ashcroft, 2004). Activated Src (either v-Src or Src with activating mutations) has been implicated in both HIF-1α stabilization and increased translation depending on the model system examined (Jiang *et al.*, 1997; Chan *et al.*, 2002; Karni *et al.*, 2002).

The protein tyrosine kinase activity of Src also rapidly increases following hypoxia, and Src−/− fibroblasts are greatly diminished in their capacity to induce VEGF expression in response to this stress (Mukhopadhyay *et al.*, 1995). In colon cancer cells with activated Src, both constitutive and hypoxia-inducible expression of VEGF are increased (Fleming *et al.*, 1997; Ellis *et al.*, 1998). As Src leads to activation of the PI3-kinase/Akt pathway in a number of tumor cells, increased VEGF expression may occur through this pathway (Alvarez-Tejado *et al.*, 2001). In addition to increasing the expression of HIF-1α, Src also regulates phosphorylation of STAT3. This phosphorylation is required for transformation of fibroblasts by v-Src, and STAT3 phosphorylation is increased in many tumor cells with high Src activity (Yu *et al.*, 1995; Cao *et al.*, 1996; Bromberg *et al.*, 1998). Recently, STAT3 has also been implicated as a positive regulator of VEGF transcription (Niu *et al.*, 2002b; Wei *et al.*, 2003). In this study, we examined the relationship between Src activation, HIF-1α expression, and STAT3 phosphorylation with respect to regulation of VEGF expression.

Our results show that incubation of cells by $CoCl_2$ (which mimics hypoxia) results in activation of STAT3 and HIF-1α, and inhibition of Src activation by the addition of the selective Src-family inhibitor, PP2, blocks this activation. Induction of activated Src (by a tet-on system) in PANC-1 and PC-3 cells increased VEGF expression, steady-state HIF-1α levels, and phosphorylation of STAT3, as was observed in cells incubated with $CoCl_2$. When cells in which activated Src had been induced were incubated with $CoCl_2$, no further significant increases in VEGF expression was observed, although we cannot discount that there may be additive or synergistic effects with $CoCl_2$ treatment (data not shown) that are undetected. These results suggest that Src activation is a significant component of hypoxia-induced expression of VEGF.

Previous studies have suggested that HIF-1α or STAT3 alone are capable of activating VEGF transcription (Niu *et al.*, 2002b; Buchler *et al.*, 2003; Stoeltzing *et al.*, 2003; Wei *et al.*, 2003). Our data suggest that hypoxia-mediated VEGF expression requires binding of both STAT3 and HIF-1α to the VEGF promoter for maxium induction in pancreatic and prostate carcinomas. As we have shown, expression of either a dominant negative STAT3 or HIF-1α reduces promoter activity significantly, suggesting that loss of binding by either transcription factor compromises VEGF expression, and may negate any synergistic effect conferred by both factors on VEGF production. In ChIP analysis on PANC-1 cells, increasing the PCR cycles from 30 to 50 showed that small amounts of HIF-1α binding to VEGF promoter prior to stimulation, while no STAT3 bound to its prospective element (data not shown). Basal transcription of the VEGF promoter has been proposed to require HIF-1α (Forsythe *et al.*, 1996; Buchler *et al.*, 2003). However, it has been suggested that maximum induction of HIF-1 target genes requires functional interactions between HIF-1 and other transcription factors (Pugh *et al.*, 1994; Ebert and Bunn, 1998; Semenza, 2003). Investigation of hypoxic activation of the LDH-A gene showed that two binding sites, an HIF and a CREB-1/ATF-1 site, are required for expression (Firth *et al.*, 1995; Ebert and Bunn, 1998). The Endothelin-1 (ET-1) gene is induced by hypoxia and also requires two tandem binding elements, an HIF-1 and an adjacent AP-1-binding site (Yamashita *et al.*, 2001). Our data provide further support that activation by HIF requires an adjacent transcription factor-binding site, and may help to explain tissue-specific differences observed in HIF-mediated induction of some transcripts by various stimuli (Semenza, 2003; Bardos and Ashcroft, 2004).

We also show that HIF-1α and STAT3 coimmunoprecipitate with CBP/p300 and Ref-1/APE, and levels of coimmunoprecipitation increase with Src activation (Figure 6). These results suggest that HIF-1α and STAT3 are components of a large complex governing transcription of VEGF. The coactivator p300 binds to a variety of transcription factors including STAT3 and HIF-1α, and is a critical component in hypoxic-regulated HIF-1α transcriptional complexes (Yuan *et al.*, 1996; Zhang *et al.*, 1996; Lill *et al.*, 1997; Ebert and Bunn, 1998; Kallio *et al.*, 1998; Paulson *et al.*, 1999; Schuringa *et al.*, 2001; Yamashita *et al.*, 2001). Gene activation by p300 is controlled in part by its ability to bind upstream transcription factors, such as those in the composite VEGF regulatory element, and coordinate this complex with the basal transcription machinery (Abraham *et al.*, 1993; Merika *et al.*, 1998; Nakashima *et al.*, 1999). Another component of the HIF-1/STAT3 transcriptional complex is Ref-1/APE (Figure 6d). Ref-1/APE is critical in oxygen-regulated gene expression. In addition to increasing the DNA affinity of transcription factors, Ref-1/APE targets the recruitment of the p300 to the transcriptional complex. Ref-1/APE facilitates DNA binding and transcriptional activity of a number of transactivating factors including HIF-1α STAT3 (Xanthoudakis and Curran, 1992; Carrero *et al.*, 2000; Ziel *et al.*, 2004), but prior to this study has not been shown to physically associate with STAT3. Src activation thus results in increased activity of HIF-1α and

STAT3, which translocate to the nucleus and in conjunction with Ref-1/APE bind to the composite regulatory element in the VEGF promoter. This complex may lead to the recruitment of p300, which serves as a platform for other components of the transcription complex, which together activate gene expression.

Finally, our results suggest one potential mechanism by which Src may contribute to tumor progression. Src activity often increases during tumor progression and may be predictive of poor prognosis (Talamonti *et al.*, 1993; Summy and Gallick, 2003). By regulating both STAT3 activation and HIF-1α expression, increasing Src activity may augment the hypoxic response in VEGF expression, itself a prognostic marker in many tumors (Hartenbach *et al.*, 1997; Niedergethmann *et al.*, 2002; Nam *et al.*, 2004; Uehara *et al.*, 2004). In addition to enhancing angiogenesis, hypoxia-mediated activation of HIF-1α and STAT3 may also contribute to increased tumorigenic properties in pancreatic and prostrate carcinomas. HIF also regulates invasion and cell survival (Semenza, 2003), while STAT3 activation is associated with increased cell proliferation and survival (Niu *et al.*, 2002a). These properties, combined with increased angiogenesis, can result in a more aggressive cancer and suggests that Src, as well as HIF-1α, STAT3, or Ref-1/APE will provide valuable molecular targets for combating the growth and metastasis of human tumors.

Materials and methods

Cell lines and cell culture conditions

The adenocarcinoma PANC-1 cell line and the prostate carcinoma PC-3 cell line were purchased from ATCC (Manassas, VA, USA) and maintained in either MEM (PANC-1) or F-12/DMEM (PC-3) supplemented with 10% FBS, 2.0 mM L-glutamine, and penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY, USA), at 37°C with 5% CO_2.

Generation of activated Src TET-on cell lines

TET-On cells lines were created using the TET-On system (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. 1×10^6 subconfluent PANC-1 and PC-3 cells were transfected with the pTet-On plasmid. DNA (1 μg) was prepared with 6 μl of Fugene 6 according to the manufacturer's directions. At 24 h after transfection, the medium was removed and cells were washed with 37°C PBS, then supplemented with complete media containing 600 μg/ml (PANC-1 cells) or 300 μg/ml (PC-3 cells) of G418 (Invitrogen/Life Technologies) for selection. Single colonies were isolated and expanded for further analyses by transient transfection of the pTRE-Luc and treated for 24 h with 2 μg/ml doxycycline. The pTRE plasmid DNA was linearized with *Bam*H1 and treated with Klenow DNA polymerase, followed by digestion with *Sal*I. A 1.8 kb Src-527 cDNA was excised from pRSV-Src527 plasmid (Kmiecik and Shalloway, 1987) with *Not*1 (followed by Klenow treatment) and *Xho*1 digestions. The cDNA was ligated into pTRE yielding pTRE-Src527. Clones of PC-3 and PANC-1 expressing the highest level of luciferase activity were cotransfected with pTRE-Src527 and selected with hygromycin B (250 μg/ ml PANC-1 and 100 μg/ml PC-3) in complete media with G418. Stable clones were screened for Src 527 expression by immunoblot analysis after 24 h doxycycline treatment using a chicken-Src-specific antibody (EC-10). Control clones cotransfected with pTRE and selected with Hygromycin B and G418 of each cell line were also obtained.

Transient expression assays

PANC-1 and PC-3 cells were maintained in culture as described. All plasmid DNA used in transfections was prepared using Qiagen plasmid DNA isolation kits according to the manufacturer's instructions. For luciferase assays, 2 days prior to transfection 0.125×10^6 cells were plated per well in 12-well plates supplemented with complete media. Immediately prior to transfection, cells had 250 μl of fresh media added to each well. All cells were transfected with a total of 1.5 μg of DNA using Fugene-6 reagent (Roche, Mannheim, Germany) following the manufacturer's protocol. DNA used was as follows: 200 ng of VEGF luciferase reporter plasmid (2.2 kb) combined with either 1.3 μg of pCEP4 (control wells), 1.3 μg of HIF-DN plasmid (Forsythe *et al.*, 1996), or 1.3 μg of STAT3-DN plasmid. Cells were washed with 37°C PBS 4 h after transfection and 2 ml of complete media was added to each well. Following a 16-h recovery period, cells were treated with doxycycline (Src tet-on system cells 0.2 μg/ml) or $CoCl_2$ (100 μM) in complete media. Following a 24-h incubation, the medium was removed and stored at −80°C for VEGF production analysis. Cells were washed in PBS and lysed in 100 μl of Promega passive lysis buffer. Luciferase activity was determined by using 20 μl of cell extract that was mixed with 100 μl of luciferase assay reagent (Promega, Madison, WI, USA) and placed in a luminometer (Monolight 2010). Light production was measured twice for 10 s and expressed as RLU, and all transfections were performed in triplicate. Relative luciferase activity was calculated as average luciferase (RLU)/ μg protein content as measured by the Bradford assay (Bio-Rad, Hercules, CA, USA).

ELISA

VEGF production in culture supernatants was examined using a human VEGF-specific ELISA (Quantikine; R&D Systems, Minneapolis, MN, USA) normalized to total protein content as measured by Bradford assay.

Immunofluorescence microscopy

At 2 days prior to transfection 5000 cells were plated per chamber in an eight-chamber slide. All cells were transfected with a total of 1.0 μg of DNA (pCEP4, HIF-1α-DN, of STAT3-DN) using Fugene-6 reagent as previously described. Following a 16-h recovery period, cells were treated with doxycycline (2 μg/ml) or PBS (control) for 24 h. Cells were then fixed with 37% formaldehyde in PBS, permeabilized in PBS containing 0.5% Triton X-100 and 1% bovine serum albumin (BSA), and blocked with 10% fetal bovine serum (FBS) in PBS. Cells were incubated with 1 : 50 anti-HIF-1α (BD Transduction Laboratories, San Jose, CA, USA) or an anti-STAT3 (Cell Signaling) antibody diluted in blocking solution. Cells were washed and incubated with fluorescein (FITC)- or Texas-Red-conjugated anti-mouse or anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) diluted 1 : 100. Nuclei were stained with Hoechst 33342 at 1 μg/ml (Molecular Probes,

Eugene, OR, USA). Cells were examined using a fluorescent microscope (Nikon Microphot-FXA).

Immunoblot assays

Cells were grown to a confluencey of 85–90% in complete media and solublized in 20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 2 mM EDTA, with 1 complete Mini Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Basel, Switzerland). Nuclear extracts were prepared by adding 1 ml of cell lysis buffer (10 mM HEPES–KOH, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCL; 0.5 mM DTT). Cells were lysed with a Dounce homogenizer (B-type pestle) at 4°C, centrifuged and the resulting pellet was resuspended in 1.5 ml of nuclear lysis buffer (20 mM HEPES–KOH, pH 7.9; 25% v/v glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM PMSF; 0.5 mM DTT) and incubated at 4°C for 30 min. Nuclear extracts were concentrated with 0.35 mg/ml of solid sodium, centrifuged and resuspended in 0.5 ml of 4°C suspension buffer (20 mM HEPES–KOH, pH 7.9; 20% v/v glycerol; 1.5 mM KCl; 0.2 mM EDTA; 0.5 mM PMSF; 1 mM DTT). Dialysis of the purified nuclear extract in 500 ml of buffer D at 4°C was preformed for 5 h followed by storage of the extract at −80°C. Immunoblot analysis of total cellular extract utilized 50 or 500 μg for immunoprecipitations, while analysis of nuclear extracts utilized 50 μg of lysates. Protein concentrations were determined by Bradford assay. Immunoprecipitations were performed in 500 μl of 20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 2 mM EDTA, 1 complete Mini Protease Inhibitor Cocktail Tablet, and 1.0 μg of 1° antibody and rotated overnight at 4°C. Antibody–Protein complexes were isolated following 2 h incubation with 25 μl of protein A/G. Lysates were fractionated by 8% SDS–PAGE, transferred to PVDF membrane (Amersham Corp., Arlington Heights, IL, USA), and probed with either anti-Phospho-Src 416, anti-STAT3, anti-Phospho-STAT3 (Ser 705) (Upstate Biotechnology Inc., Lake Placid, NY, USA), or anti-HIF-1α (BD Biosciences, Franklin Lakes, NJ, USA). Antibodies were diluted in Tris-buffered saline-0.1% Tween 20 (v/v) with 5% dried milk. Peroxidase-conjugated secondary antisera, goat anti-mouse antisera (Bio-Rad, Hercules, CA, USA), or goat anti-rabbit (Bio-Rad) were used to detect the respective primary antibodies. Immunoreactive proteins were visualized with Chemiluminescence Reagent Plus detection system (NEN, Boston, MA, USA).

ChIP assay

Cells (approx 1.0×10^{10}) were grown to a confluencey of 85–90% in complete media and treated with or without doxycycline (2 μg/ml) or 100 μM $CoCl_2$ (to mimic hypoxia) for 24 h. Cells were treated with 1% formaldehyde for 10 min at room temperature (RT), followed by the addition of 2 M glycine to a final concentration of 0.125 M. Cells were washed in 4°C PBS and pelleted in 1 ml of lysis buffer (5 mM PIPES, 85 mM KCL, 0.5% NP-40, and protease inhibitors) and incubated for 10 min at −20°C. Cells were pelleted and nuclei resuspended in 0.5 ml of nuclei lysis buffer (50 mM Tris-CL, pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitors) and incubated for 10 min at −20°C. Nuclear lysates were sonicated (Sonic Dismembrator 60, Fisher Scientific) for five 3-s intervals on ice to shear DNA to 0.800–1.2 kb fragments. Chromatin solutions were precleared with salmon sperm/protein A agarose-50% slurry (Upstate Biotech.) while rotating at 4°C for 15 min. Precleared supernatant were incubated with anti-STAT3, HIF-1α, or no Ab (negative control) and rotated overnight at 4°C. Chromatin/protein complex were isolated with 15 μl of Staph A cells. Chromatin was purified by adding 1 μl RNAase A (10 mg/ml), NaCL (0.3 M final concentration) and incubating at 67° for 6 h, followed by phenol chloroform extraction and ethanol precipitation. The region −1386 to −1036 of the VEGF promoter was PCR amplified from the immunoprecipitated chromatin using the following primers: sense 5′-CAGGTCAGAAACCAGCCAG, antisense; 5′-CGTGATGATTCAAACCTACC. The 350-bp PCR product was resolved on a 1.2% agarose gel and visualized by ethidium bromide staining and UV illumination. Positive controls for all reactions utilized chromatin sonicated as described above, and were phenol/chloroform extracted followed by ethanol precipitation.

Quantitation of autoradiograms

Autoradiographs were quantified in the linear range of the film by scanning the image using a Hewlett Packard Scanjet scanner and quantitated with Scion Image software program. Each sample measured was calculated as the ratio of the average area over the average area of actin or vinculin for immunoblots or as the ratio above the average area of basal/control levels of the protein of interest in immunoprecipitations.

Statistical analyses

Statistical analyses were performed using InStat 2.01 statistical software (GraphPad Software, San Diego, CA, USA) using the Student's *t* test, or Fisher's Exact test, where appropriate. Significance was determined with 95% confidence.

Abbreviations

VEGF, vascular endothelial growth factor; STAT3, signal and transducer of transcription 3; HIF, hypoxia inducible factor.

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